

# METHOD FOR PREVENTING SUPEROXIDE DAMAGE TO CELLS AND OXYGEN-LABILE PROTEINS

## CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to provisional patent application Serial No. 60/234,588, filed September 22, 2000, incorporated by reference herein.

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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## BACKGROUND OF THE INVENTION

[0002] Cell physiology is characterized by the interplay of numerous metabolic pathways and processes. Integration is essential to create a metabolism that is robust, yet adaptable to complex environmental conditions, such as growth in the presence or absence of oxygen. Although aerobic respiration provides a substantial energetic advantage, it necessarily generates toxic oxygen species that can damage macromolecules (Gonzalez-Flecha, B. and Demple, B., J. Biol. Chem. 270:13681-7, 1995; Imlay, J. A. and Fridovich, I., J. Biol. Chem. 266:6957-65, 1991. For example, superoxide radicals ( $O_2^-$ ) can oxidize labile [4Fe-4S] to inactive [3Fe-4S] clusters (Flint, D. H., et al., J. Biol. Chem. 268:22369-76, 1993; Kuo, C. F., et al., J. Biol. Chem. 262:4724-7, 1987). Such oxidation has at least two detrimental consequences, inactivation of enzymes containing [Fe-S] clusters (Flint, D. H., et al., supra, 1993; Gardner, P. R. and Fridovich, I., Arch. Biochem. Biophys. 284:106-11, 1991; Gardner, P. R. and Fridovich, I., J Biol Chem 266:1478-83, 1991; Gardner, P. R. and Fridovich, I., J. Biol. Chem. 266:19328-33, 1991), and increased DNA damage (Imlay, J. A. and Linn, S., Science 240:1302-9, 1988; Keyer, K. and Imlay, J. A., Proc. Natl. Acad. Sci. USA 93:13635-40, 1996). DNA damage results from ferrous ions, released during the oxidation of [4Fe-4S] clusters. These ions

participate in Fenton chemistry ( $\text{Fe(II)} + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{Fe(III)} + \text{H}_2\text{O} + \text{OH}\bullet$ ), with the hydroxyl radicals damaging DNA and other macromolecules (Keyer, K. and Imlay, J. A., supra, 1996; Liochev, S. I. and Fridovich, I., Free Radic. Biol. Med. 16:29-33, 1994; Srinivasan, C., et al., J. Biol. Chem. 275:29187-92, 2000). It would not be surprising that many cellular anomalies caused by increased superoxide concentration result from oxidization of [Fe-S] clusters (Keyer, K. and Imlay, J. A., supra, 1996).

[0003] Several systems exist to reduce the potential for damage by superoxide radicals (Storz, G. and Imlay, J. A., Curr. Opin. Microbiol. 2:188-94, 1999). In general, these systems either prevent the damage from occurring or repair it. The Sox regulon is a good example of the former. This regulon includes a number of genes that are induced under conditions of oxidative stress via the SoxRS regulatory system (Hidalgo, E. and Dimple, B., Embo J. 16:1056-65, 1997; Gaudu, P., et al., J. Biol. Chem. 272:5082-6, 1997; Liochev, S. I., et al., J. Biol. Chem. 274:9479-81, 1999). One component of this system is the superoxide dismutase enzymes (SOD, EC 1.15.1.1) that catalyze the formation of molecular oxygen and hydrogen peroxide from two superoxide radicals ( $\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$ ). The resulting hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is a substrate for catalase (hydroperoxidase) enzymes (EC 1.11.1.6, 1.11.1.7) that convert it to water and molecular oxygen. A distinct way of protecting [Fe-S] clusters is shown by the FeSII protein of *Azotobacter vinelandii*. The FeSII, or Shetna protein, forms a complex with nitrogenase under periods of high oxygen exposure, thus protecting the essential [Fe-S] cluster from oxidation (Lou, J., et al., Biochemistry 38:5563-71, 1999; Shethna, Y. I., et al., Biochem. Biophys. Res. Commun. 31:862-8, 1968).

[0004] In addition to eliminating superoxide *per se*, mechanisms to repair damage incurred by the superoxide radicals have evolved. This second strategy includes multiple repair systems that are specific for DNA damage (McCullough, A. K., et al., Annu. Rev. Biochem. 68:255-85, 1999; Cadet, J., et al., Mutat. Res. 462:121-8, 2000; Boiteux, S. and Radicella, J. P., Biochimie 81:59-67, 1999). The DNA glycosylase MutY, which itself contains an [Fe-S] cluster (Michaels, M. L., et al., Nucleic Acids Res. 18:3841-5, 1990; Porello, S. L., et al., Biochemistry 37:6465-75, 1998), recognizes the mispairing of an oxidized guanine base (8-oxo-guanine) with adenine and cleaves the relevant adenine (Michaels, M. L., et al., Biochemistry 31:10964-8, 1992). This cleavage product becomes the target for additional repair enzymes that prevent the generation of a G•C to T•A transversion mutation.

[0005] Another example involves direct repair of oxidized [Fe-S] clusters *in vivo*. The enzyme paradigm for the majority of studies addressing the *in vivo* and *in vitro* reconstitution of [Fe-S] clusters is aconitase (Acn, EC 4.2.1.3) (Kennedy, M. C. and Beinert, H., J. Biol. Chem. 263:8194-8, 1988; Gardner, P. R. and Fridovich, I., J. Biol. Chem. 267:8757-63, 1992; Gardner, P. R. and Fridovich, I., Arch. Biochem. Biophys. 301:98-102, 1993). Part of the catalytic [4Fe-4S] center in aconitase is exposed to the solution and is not sequestered by the enzyme; thus the enzyme is sensitive to attack by superoxide (Gardner, P. R. and Fridovich, I., supra, 1992; Beinert, H., et al., Chem. Rev. 96:2335-2373, 1996). Although extensive work has been performed to characterize *in vitro* reactivation of oxidized [Fe-S] clusters (Kennedy, M. C. and Beinert, H., supra, 1988), the participants in [Fe-S] cluster repair *in vivo* are less well defined (Gardner, P. R. and Fridovich, I., supra, 1993). The benefit of *in vivo* repair of [Fe-S] clusters is at least two fold, first the restoration of enzyme activity, and second, the decrease of free iron.

[0006] Several experiments have suggested that glutathione (GSH) is involved in the *in vivo* repair and possibly biosynthesis, of the [Fe-S] center in aconitase (Gardner, P. R. and Fridovich, I., supra, 1993). When *Escherichia coli* strains *in vivo* were challenged with oxygen total aconitase activity decreased, as expected for an enzyme with a labile [Fe-S] cluster. However, when the oxygen challenge was removed, unlike the wild-type strain, *gshA* (encodes  $\gamma$ -l-glutamyl-l-cysteine synthetase, EC 6.3.2.2) mutants were unable to regain aconitase activity in the absence of protein synthesis (Gardner, P. R. and Fridovich, I., supra, 1993). Further, *gshA* mutants of *E. coli* have reduced total aconitase activity (Gardner, P. R. and Fridovich, I., supra, 1993).

[0007] Needed in the art is an improved method of protecting cells and oxygen-labile enzymes from superoxide damage.

#### BRIEF SUMMARY OF THE INVENTION

[0008] We disclose herein that increased levels of the YggX protein reverse several metabolic defects attributed to a lack of GSH, increase resistance to superoxide stress, and decrease the spontaneous mutation frequency in *S. enterica*. The phenotypic consequences of increased YggX protein are consistent with a model in which this protein protects labile [Fe-S] clusters from oxidative damage.

[0009] In one embodiment, the present invention is a method of reducing superoxide damage to a cell, comprising the step of engineering the cell to produce more than the native amount of the YggX protein or its homolog, wherein the cells are rendered more resistant to oxidative damage. A preferred method additionally comprises the step of analyzing the protein to determine that the cells are rendered more resistant to superoxide damage.

[0010] Another embodiment of the present invention comprises increasing the resistance of oxygen-labile proteins to oxidative damage, comprising the step of co-expressing the oxygen-labile protein with the YggX protein or a homolog of the YggX protein. Preferably, one additionally examines the oxygen-labile protein to determine the amount of superoxide damage.

[0011] Another embodiment of the present invention comprises a method of screening compounds for antibiotic properties, comprising the step of examining a test compound's ability to effect YggX activity or the activity of a YggX homolog, wherein decreased YggX activity indicates that the compound is a likely candidate as an antibiotic.

[0012] It is an object of the present invention to protect cell and oxygen-labile proteins from superoxide damage.

[0013] Other objects, features and advantages of the present invention will become apparent to one of skill in the art after review of the specification, claims and drawings.

#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0014] Fig. 1. Physical parameters of *yggX* and its gene product. (A) Alignment of YggX homologs. (B) Operon structure of *mutY / yggX* in *E. coli* and *S. enterica* LT2. Promoters were mapped by Gifford and Wallace in *E. coli* (Gifford, C. M. and Wallace, S. S., J. Bacteriol. 181:4223-36, 1999).

[0015] Fig. 2. Increased levels of YggX protein in *yggX*\* mutant. Western blot analysis was performed according to Harlow and Lane (Harlow, E. and Lane, D. (1988) *Antibodies* (Cold Spring Harbor Laboratory, USA). Proteins were visualized using alkaline phosphatase conjugated to anti-rabbit secondary antibody (Promega, Madison, WI). Lanes A, B and C were loaded with crude cell-free extracts (1 µg

protein) from strains DM5104, DM5105 (*yggX*<sup>\*</sup>) and DM5647 (*yggX*::Gm), respectively. Lane D was loaded with 1 ng purified YggX.

[0016] Fig. 3. The *yggX*<sup>\*</sup> mutation does not increase MNNG resistance of *gshA* mutants. Strain LT2 was grown in LB with (▲) and without (Δ) 60 μM MNNG. Both *gshA* (○) and *gshA yggX*<sup>\*</sup> (●) mutant strains were grown in LB with 60 μM MNNG.

[0017] Fig. 4. The *yggX*<sup>\*</sup> mutation increases resistance of *S. enterica* to PQ. Panel A shows growth of *gshA* (○) and *gshA yggX*<sup>\*</sup> (●) mutant strains in LB with 4 μM PQ. Panel B shows growth of LT2 (Δ) and *yggX*<sup>\*</sup> (▲) strains in LB with 40 μM PQ.

[0018] Fig. 5. *yggX*<sup>\*</sup> does not require *soxR* to mediate resistance to PQ. Strains LT2 (◆), *soxR* (◇) and *soxR yggX*<sup>\*</sup> (▼) were grown in LB with 4.0 μM PQ.

[0019] Fig. 6. Model showing how YggX protects *S. enterica* from oxidative damage. The result of superoxide attack on [Fe-S] clusters is depicted. We hypothesize that YggX is able to block oxidative damage to labile clusters and thus prevents the normal downstream consequences of such oxidation.

#### DETAILED DESCRIPTION OF THE INVENTION

[0020] In brief, the present invention involves the use of YggX, a protein identified from *Salmonella enterica* Seroovar Typhimurium, or the homolog of this protein to protect cells or oxygen-labile enzyme from oxidated damage. By "homolog" we mean a protein with a function substantially identical to the *Salmonella typhimurium* YggX protein with at least a 45%, preferably 55%, amino acid identity. Fig. 1A compares the YggX homologs of various bacterial species. Of the 17 invariable amino acids, a homolog suitable for the present invention will comprise at least 14 and preferably all 17. We have compared a number of sequences and have found

that 14 of the residues are invariant throughout all sequences examined. The conserved motif of suitable homologs is

[0021] MXRXXXCXXX XXXXXXXXXXXX XXPXXXGXXX XXXXXXXXWX  
XWXXXQTXLX NEXXLXXXXX XXRXX, wherein X is any amino acid.

[0022] These 14 residues are represented by dark shading in Fig. 1A. A preferred sequence of the present invention will comprise these 14 invariant residues and will be approximately the same size as the YggX protein.

[0023] In one aspect of the present invention, one would overexpress the YggX protein or its homolog in a cell to provide resistance to superoxide damage. (By "overexpress", we mean that the protein will be expressed at greater than native levels.) One would preferably first amplify the YggX gene from the bacterial chromosome and ligate the gene into a standard expression vector suitable for the strain to be protected. One would use the expression of the YggX gene and, preferably, examine the strain to determine resistance to oxidative damage. Resistance to oxidative damage would preferably be determined by the ability of the cell to grow in an increased concentration of super oxide producing compounds (e.g. paraquat) compared to the cell with lower levels of YggX protein.

[0024] In another embodiment of the present invention, one would co-express the YggX protein or its homolog with an oxygen-labile protein, preferably one with an iron sulfur cluster center. In this manner, one would protect the particular protein from superoxide damage. If one wished to co-express YggX to stabilize an oxygen-labile protein, one would first amplify the *yggX* gene from a bacterial chromosome that contains a homolog using standard PCR techniques. One would then ligate the *yggX* gene into a standard expression vector and transform the *yggX* expression vector into a strain that can express the oxygen-labile protein of interest. When

inducing the strain to express the protein of interest, one would also induce expression of the *yggX* gene. The "stabilization" of oxygen-labile proteins would preferably be measured by detecting increased activity of an oxygen-labile protein or by recovering increased yield of the active protein. Preferred oxygen-labile proteins are those containing at least one [FE] cluster.

[0025] While we have performed experiments thus far in bacterial cells we anticipate a similar mechanism of protection to occur with YggX in other cell types, including yeast, mammalian and plant cells. This expectation is due to the similarity of structure, function and oxygen lability of [Fe-S]-containing proteins in each of these cell types.

[0026] One could obtain the YggX protein through standard molecular biology techniques and reference to Gralnick and Downs, Proc. Natl. Acad. Sci. 98(14):8030-8035, 2001, incorporated by reference. Applicants note that the amino acid sequence of the E.Coli YggX is listed at GenBank accession number AAC75999.

[0027] In another aspect of the present invention, one would use the YggX protein or its homologs as the target for antibiotics. In one embodiment, one would examine test compounds to determine whether the compound affected the activity of the YggX protein. Successful compounds would make excellent candidates for antibiotics.

[0028] Successful compounds that affect the activity of YggX could be identified as those that reverse the growth advantage (i.e., increased resistance to paraquat) that is allowed by increased levels of YggX protein. One would develop an *in vitro* assay for YggX, and these compounds would be expected to alter the kinetic parameters of YggX in the assay.



## EXAMPLES

[0029] We disclose herein that elevated levels of the YggX protein increase the resistance of *Salmonella enterica* to superoxide stress, reverse enzymatic defects attributed to oxidized [Fe-S] clusters, and decrease the spontaneous mutation frequency. The data are consistent with a model in which YggX protects protein [Fe-S] clusters from oxidation.

### Materials and Methods

[0030] Strains, media and DNA manipulations. Strains used were derivatives of *S. enterica* Serovar Typhimurium strain LT2. Media, antibiotics, and insertion nomenclature have been described previously (Gralnick, J., et al., J. Bacteriol. 182:5180-7, 2000). All chemicals were purchased from Sigma Chemical Co.

[0031] Enzymes for DNA manipulations were purchased from Promega and used as per the manufacturer's instructions. Sequencing was carried out by the University of Wisconsin Biotechnology Center. PCR amplification of *S. enterica yggX* used *E. coli* ORFmers (b2962-A, b2962-C) with conditions specified by the manufacturer (Sigma-Genosys).

[0032] Genetics i) Transduction. The methods of transduction using P22 (HT105/1, *int-201*) (Schmieger, H., Mol. Gen. Genet. 119:75-88, 1972) and purification of transductants has been described (Downs, D. M., J. Bacteriol. 174:1515-21, 1992).

[0033] ii) Isolation of mutants overexpressing YggX (yggX\*). Cells from an overnight nutrient broth (NB) culture were pelleted, washed twice with NaCl (85 mM), and aliquots plated on minimal glucose medium. Colonies arose after 2 – 3 days of 37°C incubation. A Tn10d (Cm) insertion (Way, J. C., et al., Gene 32:369-79, 1984) linked to the causative mutation was identified by standard genetic techniques (Kleckner, N., et al., J. Mol. Biol. 116:125-59, 1977).

[0034] *iii) Identification of yggX locus.* Genomic DNA from a suppressed *gshA* strain was partially digested with *Sau*3A, ligated into vector pSU19(Cm), and the resulting DNAs were electroporated into LT2 cells. Electroporants were selected for Cm<sup>R</sup> and screened for increased resistance to paraquat PQ (100 µL 0.1% PQ spread on a NB plate). Plasmid DNA was isolated, electroporated into strains DM271 (*apbE*) and DM4620 (*gshA*) and prototrophy scored. One clone, pPQR4 (Fig. 1), satisfied all requirements, and was used further.

[0035] *iv) Generation of chromosomal yggX insertion.* Plasmid pYGGX3A::Gm was transduced into a *polA*-deficient strain (DM3961). The transduction was allowed to proceed for 1 hour, cells were then washed twice in LB + 5 mM EGTA and incubated at room temperature overnight prior to spreading onto NB/Gm plates. Colonies that arose on NB/Gm plates were screened for Cm<sup>S</sup>, indicating loss of the parent plasmid by a double crossover event. The Gm<sup>R</sup> cassette from Gm<sup>R</sup>Cm<sup>S</sup> strains was transduced into wild type LT2; the insertion in *yggX* was confirmed by PCR amplification.

[0036] *v) Strain construction.* A *soxR* deletion strain of *Escherichia coli* (DJ901) was obtained and the marker (*zjc-2204::Tn10* (Km)) linked to the deletion (Greenberg, J. T., et al., Proc. Natl. Acad. Sci. USA 87:6181-5, 1990) was transduced into *S. enterica* LT2 via a *mutS* intermediate as described (O'Brien, K., et al., Gene 11813-9, 1992; Beck, B. J., et al., J. Bacteriol. 179:6504-8, 1997). Transductants were scored for the  $\Delta$ *soxR*901 allele (sensitivity to 4 µM paraquat, (PQ)). An isogenic pair of strains with (DM5317) and without (DM5319) the  $\Delta$ *soxR*901 allele was constructed. The presence of the *yggX*\* mutation in relevant strains was confirmed by backcross into a *gshA* strain (DM4620).

- [0037] *vi) Nutritional requirements.* Nutritional requirements were tested with solid medium, soft agar overlays and growth curves in micro-titer plates (Petersen, L., et al., Genetics 143:37-44, 1996; Christian, T. and Downs, D. M., Can. J. Microbiol. 45:565-72, 1999).
- [0038] *vii) Spontaneous mutation frequency.* Cultures were grown by shaking overnight in LB at 37°C. Aliquots (100–200 µl) were plated on solid LB media containing 100 µg/ml rifampacin, and incubated overnight at 37°C. In the case of d-cycloserine resistance, cultures were grown overnight in defined medium. Aliquots (10–100 µl) were plated on minimal glucose plates containing 0.2 mM d-cycloserine (0.2 M stock in phosphate buffer pH 8.0), and incubated overnight at 37°C. Colony-forming units (CFUs) were determined by plating on non-selective media.
- [0039] Enzyme assays. *i) Aconitase.* Aconitase activity was assayed in cell-free crude extracts by the protocol of Gruer and Guest (Gruer, M. J. and Guest, J. R., Microbiology 140:2531-41, 1994), as modified by Skovran (Skovran, E. and Downs, D. M., J. Bacteriol. 182:3896-903, 2000). Specific activity was described in Units/mg protein where a unit was the change in absorbance at 240 nm per minute. Protein concentration was determined by the Bradford Assay (Bradford, M. M., Anal. Biochem. 72:248-54, 1976).
- [0040] *ii) Superoxide Dismutase (EC 1.15.1.1).* SOD assays were modified from McCord and Fridovich (McCord, J. M. and Fridovich, I., J. Biol. Chem. 244:6049-55, 1969). Cultures (5 mL of LB grown overnight at 37°C) were washed once with 3 mL 50 mM KH<sub>2</sub>PO<sub>4</sub>/0.1 mM EDTA, then resuspended in 1 mL of this buffer. Cells were kept on ice and sonicated 3 times 10 seconds (0.5 second pulses, power set to 3) using a Sonic Dismembrator 550 (Fischer Scientific). Extracts were centrifuged to

remove cell debris and unbroken cells, and kept on ice until assayed. A unit of SOD activity was as described (McCord, J. M. and Fridovich, I., supra, 1969).

[0041] YggX overexpression and purification. The *yggX* gene was cloned into the *NdeI* and *SmaI* sites of the pTYB2 expression vector (New England Biolabs) contained in the IMPACT T7 Kit. The resulting plasmid, pJAG100, was electroporated into strain BL21( $\lambda$ DE3). Overexpression and purification were performed per manufacturer's recommendations, with the exception that the dithiothreitol (DTT) concentration used during the on-column cleavage step was 50 mM. Protein was concentrated using an Ultrafree-15 centrifugal filter device (Millipore Corporation) with a 5K MW cutoff. Anti-YggX polyclonal rabbit antibodies against purified YggX were generated at the University of Wisconsin Animal Care Unit.

## Results

[0042] A suppressor of *gshA* mutant phenotypes. We recently demonstrated that *gshA* mutants of *Salmonella typhimurium* serovar Typhimurium strain LT2 are thiamine auxotrophs (Grainick, J., et al., supra, 2000). When a *gshA* mutant strain was incubated on minimal glucose plates for 2–3 days, colonies arose at a frequency of  $\sim 10^{-5}$ . Genetic analyses of 10 independent colonies demonstrated that prototrophic growth resulted from a single lesion. An insertion (*zgf*-8077::Tn10d(Cm)) was 80% linked by P22 transduction to the causative mutation in each of the 10 revertants. The suppressing allele was designated *yggX*<sup>\*</sup>, to be consistent with annotation of the *E. coli* genome.

[0043] An intact *yggX* locus is required for suppression. A plasmid library was generated using genomic DNA from a *gshA yggX*<sup>\*</sup> double-mutant strain (DM5015). Assuming the *yggX*<sup>\*</sup> mutation was dominant, clones were screened for ability to

confer PQ resistance (see below), and prototrophic growth to strain DM4620 (*gshA*). One such plasmid (pPQR4) is diagrammed in Fig. 1, and was further characterized. Sequence analysis determined that plasmid pPQR4 contained two full genes (*yggX*, *mltC*), and part of a third (*mutY*). Since additional independent clones also carried *yggX*, the involvement of this gene in prototrophic growth was pursued. A DNA fragment containing *yggX* and reduced flanking sequences was PCR-amplified from pPQR4 and used to generate plasmid pYGGX3A (Fig. 1). This plasmid conferred the same growth phenotype as pPQR4, establishing the sufficiency of the *yggX* gene for suppression.

[0044] To investigate the role of *yggX* in the growth phenotype, a targeted null mutation was generated. A cassette encoding gentamycin resistance (Schweizer, H. D., Biotechniques 15:831-4, 1993) was engineered into a unique *Bgl*II site in the *yggX* coding sequence on plasmid pYGGX3A. The resulting plasmid, pYGGX3A::Gm, failed to restore growth of strain DM4620 (*gshA*) on minimal glucose medium. When the chromosomal *yggX*::Gm insertion was transduced into strain DM5015 (*yggX*\* *gshA*), the suppression of the thiamine requirement was lost. We concluded that an intact *yggX* locus was required for the phenotypic suppression caused by a *yggX*\* mutation. No nutritional requirement was detected for the single *yggX* null mutant (data not shown).

[0045] Increased expression of *yggX* is sufficient for phenotypic suppression of *gshA* mutants. Three results led to the conclusion that the *yggX*\* mutation results in increased levels of YggX protein that cause the phenotypes attributed to this mutation. First, there were no differences in the *yggX* coding sequence between wild-type and *yggX*\* strains. Second, ORFmers were used to amplify the *yggX* coding sequence from wild-type and *yggX*\* mutant strains and generate plasmids

containing only the *yggX* coding region in each of two orientations. Only the two plasmids with inserts properly oriented with respect to the plasmid encoded *lac* promoter restored prototrophic growth of the *gshA* mutant.

[0046] Third, Western-blot analyses of cell-free extracts showed that strain DM5105 (*yggX*<sup>\*</sup>) had increased levels of YggX protein (11 kDa) compared to the isogenic strain DM5104 (Fig. 2). In fact, YggX was not detectable in the wild-type strain by this assay. The above results demonstrated that increasing the levels of YggX was sufficient to cause the phenotypes associated with the *yggX*<sup>\*</sup> mutation and they were consistent with the *yggX*<sup>\*</sup> mutation affecting expression of *yggX*.

[0047] The *yggX* gene is located at minute 66 on the *E. coli* and *S. enterica* chromosomes. In a number of organisms, *yggX* is located adjacent to *mutY* (encoding adenine DNA glycosylase), and at least in *E. coli*, these genes appear to be co-transcribed (Gifford, C. M. and Wallace, S. S., J. Bacteriol. 181:4223-36, 1999). The gene organization of *mutY* and *yggX* appears to be conserved in at least 17 out of the 23 eubacteria. We have not found *yggX* sequences in any archeal or eukaryotic genome sequences available in the GenBank Database at NCBI.

[0048] Increased level of YggX does not act by increasing the cellular levels of free thiols. Inactivation of *gshA* results in loss of GSH, the predominant free thiol in the cell (Apontoweil, P. and Berends, W., Biochim. Biophys. Acta 399:10-22, 1975). Since the phenotypes of a *gshA* mutant must be explained in the context of this loss, it was conceivable that the phenotypic suppression by *yggX*<sup>\*</sup> could be due to either *gshA*-independent formation of GSH, or elevation of a distinct free-thiol pool. The results of two experiments eliminated both of these possibilities. First, GSH levels of 14.0 pmol/mg wet weight were detected in wild-type strain (LT2) using a glutathione

cycling assay (Anderson, M. E., Methods Enzymol. 113:548-55, 1985), yet no GSH (<0.1 pmol) was detectable in either *gshA* or *gshA yggX\** mutant strains (DM5014 and 5015, respectively). Second, the *yggX\** mutation did not alter the sensitivity of a *gshA* mutant to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). MNNG is a common mutagen whose toxicity is accelerated by the presence of free thiols in the cell (Lawley, P. D. and Thatcher, C. J., Biochem. J. 116:693-707, 1970). Growth analyses were performed in the presence of 60 µM MNNG and the results are presented in Fig. 3. As expected, strain DM5014 (*gshA*) was significantly more resistant to MNNG than wild-type strain LT2 (Kerklaan, P., et al., Mutat. Res. 122:257-66, 1983), and the *yggX\** mutation had no deleterious affect on this resistance. In fact the *gshA yggX\** double mutant (DM5015) appeared to have a slightly increased growth rate. A general stimulation of growth rate was observed in several strains containing the *yggX\** mutation or the overexpression plasmid, and was attributed to the general effect of increased levels of YggX on distinct areas of metabolism described below. The resistance of *gshA yggX\** double mutants to MNNG suggested that an increased level of YggX did not elevate the pool size of a free thiol.

[0049] The breadth of phenotypes suppressed by increased levels of YggX suggests a role for this protein in protecting [Fe-S] clusters. Mutants defective in *gshA* belong to a recently defined class of thiamine auxotrophs that share several phenotypic similarities (Gralnick, J., et al., supra, 2000; Skovran, E. and Downs, D. M., supra, 2000) including a requirement for the thiazole moiety of thiamine that can be eliminated by anaerobic growth. It has been proposed that this defect reflects an inability to repair the oxidized [Fe-S] cluster in the ThiH biosynthetic enzyme (Gralnick, J., et al., supra, 2000). Although the function of their gene products has

not been determined, lesions in *apbC* (Petersen, L. A. and Downs, D. M., J. Bacteriol. 179:4894-900, 1997) and *apbE* (Beck, B. J. and Downs, D. M., J. Bacteriol. 180:885-91, 1998; Beck, B. J. and Downs, D. M., J. Bacteriol. 181:7285-90, 1999) result in a thiamine phenotype similar to that caused by a *gshA* mutation. The effect of the *yggX*\* mutation on thiamine-independent growth in these mutant strains was quantified, and data from representative experiments are shown in Table 1. The data showed that the requirement for thiamine was eliminated by a *yggX*\* mutation in a strain defective in *gshA*, *apbC*, or *apbE* (Table 1, lines 2-7). These results were consistent with thiamine synthesis in these mutant strains being disrupted by a similar mechanism.

Table 1. *yggX*\* mutation eliminates thiamine requirement of *gshA* mutants

Line	Strain	Relevant genotype	Growth rate, $\mu$	
			Minimal	Min + Thi
1	LT2	Wild type	0.45	0.47
2	DM5014	<i>gshA</i>	0.11	0.32
3	DM5015	<i>gshA yggX</i> *	0.46	0.35
4	DM5784	<i>apbE</i>	0.09	0.31
5	DM5783	<i>apbE yggX</i> *	0.44	0.42
6	DM1774	<i>apbC</i>	0.20	0.37
7	DM1773	<i>apbC yggX</i> *	0.46	0.45

Specific growth rate was determined by using  $\mu = \ln(X/X_0)/T$ , where  $X$  is  $Abs_{650}$  during the log portion of the growth curve and  $T$  is time. Numbers shown are representative of at least two experiments.

Mutations in the *isc* gene cluster of *S. enterica* (Skovan, E. and Downs, D. M., supra, 2000) and *E. coli* (Schwartz, C. J., et al., Proc. Natl. Acad. Sci. USA 97:9009-14, 2000; Lauhon, C. T. and Kambampati, R., J. Biol. Chem. 275:20096-103, 2000) cause a number of metabolic phenotypes, two of which are relevant here. A polar mutation in *iscA* caused a requirement for thiazole similar to that described for the class of mutants discussed above (Skovan, E. and Downs, D. M., supra, 2000). This requirement was eliminated by the presence of either the *yggX*\* mutation or plasmid pYGGX3A (data not shown). Further, the nicotinic acid requirement generated by lack of the *iscS* gene was eliminated by the overexpression of YggX (Skovan, E. and Downs, D. M., supra, 2000), unpublished results). The nicotinic acid requirement



can be traced back to a reduced activity of NadA (quinolinic synthetase) (Skovan, E. and Downs, D. M., supra, 2000; Zhu, N. in Biology (Thesis, University of Utah, Salt Lake City, 1990, an enzyme that also contains an oxygen-labile [Fe-S] center (Gardner, P. R. and Fridovich, I., supra, 1991).

[0051] The emerging correlation between increased YggX levels and activity of [Fe-S] proteins prompted us to address aconitase activity. In both *E. coli* (Gardner, P. R. and Fridovich, I., supra, 1993) and *S. enterica* (Gralnick, J., et al., supra, 2000), *gshA* mutants have reduced total aconitase activity. This loss in activity was suggested to reflect an inability to repair the oxidized [Fe-S] center of Acn in the absence of GSH (Gardner, P. R. and Fridovich, I., supra, 1993). The specific activity of aconitase in cell-free extracts of wild-type, *gshA* and *gshA yggX\** mutant strains was  $3.50 \pm 0.32$ ,  $1.23 \pm 0.22$ , and  $3.66 \pm 0.23$  Units/mg protein, respectively.

[0052] Increased levels of YggX restored activity of at least two enzymes when assayed nutritionally (ThiH, NadA) and one when assayed biochemically (Acn). The ability of the *yggX\** mutation to completely restore Acn activity makes it feasible that suppression of the nutritional requirements reflects a significant change in the relevant enzyme activities. Experiments below identified additional metabolic consequences of increased levels of YggX, all of which could be accounted for by a model in which YggX was either limiting oxidation of [Fe-S] centers and/or facilitating their repair.

[0053] Increased levels of YggX result in *soxR*-independent resistance to superoxide. Strains carrying the *yggX\** mutation, or the expression plasmids described above, displayed increased resistance to superoxide. Supplementing the growth medium with the redox-cycling herbicide paraquat (PQ) increased the concentration of superoxide (Hassan, H. M., Methods Enzymol. 105:523-32, 1984).

Fig. 4 illustrates the effect of the *yggX*<sup>\*</sup> mutation on the growth of four strains in the presence of PQ. Data in Fig. 4A show that wild-type *S. enterica* grew slowly in the presence of 40  $\mu$ M PQ, and that a *yggX*<sup>\*</sup> mutation restored rapid growth. A *gshA* mutant was sensitive to the presence of 4  $\mu$ M PQ, as shown in Fig. 4B (Gralnick, J., et al., supra, 2000), and the *yggX*<sup>\*</sup> mutation improved growth, restoring it to a wild-type rate. In other experiments using phenazine methosulfate (PZ, 16  $\mu$ M) as the generator of superoxide, similar trends were seen. In a representative experiment, the specific growth rates of a *gshA* (DM5014) and a *gshA yggX*<sup>\*</sup> mutant strain (DM5015) in LB containing PZ were 0.15 and 0.51, respectively.

[0054] Growth in PQ induces expression of genes in the *soxRS* regulon, currently the best understood system to combat superoxide stress (Liochev, S. I., et al., supra, 1999). To test if the increased resistance of the *yggX*<sup>\*</sup> mutants to PQ was mediated through the *soxRS* regulon, various strains with lesions in *soxR* were constructed and analyzed. Some of the data from these experiments are shown in Fig. 5. In our system, as in others, a *soxR* mutant (DM5317) was more sensitive to PQ than the isogenic *soxR*<sup>+</sup> strain (DM5319). The growth data showed that a *yggX*<sup>\*</sup> mutation significantly increased the resistance of the *soxR* strain to PQ (0.4  $\mu$ M), but was unable to restore resistance to the wild-type level. We observed that a *yggX*<sup>\*</sup> mutation restored prototrophic growth to a *gshA* mutant strain, even in the presence of the *soxR* mutation (data not shown). Together, these results indicated that the resistance to PQ allowed by increased levels of YggX was not mediated through the *soxRS* system. Since inactivation of enzymes containing labile [Fe-S] centers contributes to the lethality of PQ, these results were also consistent with a model in which YggX protects [Fe-S] centers from oxidation.

[0055] It was formally possible that YggX overexpression increased the cellular level of SOD activity independent of the *soxRS* system. When SOD activity of the wild-type (DM5104) and *yggX*\* mutant strain (DM5105) were measured to address this possibility, they were found to be  $6.78 \pm 0.49$  and  $6.61 \pm 0.49$  units, respectively.

[0056] Increased levels of YggX result in a decreased frequency of spontaneous mutations. A role for YggX in mutagenesis was explored for two reasons. First, the conserved location of *yggX* adjacent to *mutY* raised the possibility that YggX was associated with MutY function. It was intriguing that MutY itself contains an [Fe-S] center, while it functions under conditions of oxidative stress in the repair of oxidatively damaged DNA (Boiteux, S. and Radicella, J. P., supra, 1999; Michaels, M. L., et al., supra, 1990; Michaels, M. L., et al., supra, 1992). In a more general context, our working model suggests that YggX reduces the oxidation of [Fe-S] clusters (see below). Thus, YggX would reduce the loss of Fe(II) ions from clusters. The resulting decrease in free-iron levels would generate fewer hydroxyl radicals and thus reduce DNA damage (Keyer, K. and Imlay, J. A., supra, 1996). As an initial test of this aspect of the model, the frequency of spontaneous mutants acquiring resistance to rifampicin or d-cycloserine was determined in several strains. Representative data for these two assays of mutation frequency are shown in Table 2. As shown by the data in Table 2, in an otherwise wild-type background, the *yggX*\* mutation reduced the number of spontaneous mutations by greater than ten-fold. As predicted by our working model, a *gshA* mutant displayed an increased mutation frequency. When the *yggX*\* mutation was present in the *gshA* mutant background, the frequency of R<sup>f</sup> colonies was decreased from 176 to a background level of 1-2 /10<sup>8</sup>. A similar trend was noted in the frequency of spontaneous mutants resistant to d-cycloserine.

## Discussion

[0057] This work was initiated to characterize a frequent mutation that suppresses the requirement of a class of thiamine auxotrophs (Gralnick, J., et al., supra, 2000). Molecular analysis found the causative mutation, *yggX\**, increased the level of the YggX protein. Overexpression of the *yggX* gene was found to alter several metabolic processes “unrelated” to thiamine synthesis. The phenotypes resulting from YggX overexpression are broad enough to suggest a role for this protein in a central metabolic process. Our working model holds that YggX protects labile [Fe-S] clusters from attack by oxygen species, including superoxide.

[0058] Fig. 6 depicts the consequences of superoxide radicals relevant to our model for the function of YggX. Superoxide (and/or other oxygen species) attack the labile [Fe-S] centers in a number of proteins (e.g., aconitase) (Flint, D. H., et al., supra, 1993; Gardner, P. R. and Fridovich, I., supra, 1991; Gardner, P. R. and Fridovich, I., supra, 1991; Gardner, P. R. and Fridovich, I., supra, 1992; Flint, D. H., et al., J. Biol. Chem. 268:14732-42. 1993). This molecular attack results in inactivation of the respective enzymes, and release of both free-iron and hydrogen peroxide that generates DNA damaging hydroxyl radicals via Fenton chemistry (Keyer, K. and Imlay, J. A., supra, 1996; Liochev, S. I. and Fridovich, I., supra, 1994; Srinivasan, C., et al., supra, 2000). It was suggested that in a wild-type cell, glutathione minimizes the effects of oxidation damage by providing reductant to facilitate reconstitution of the [Fe-S] clusters (Gardner, P. R. and Fridovich, I., supra, 1993), completing a cycle of damage and repair to the [Fe-S] clusters that remains in equilibrium under normal growth conditions. When GSH is absent (e.g., a *gshA* mutant), the effects of these oxygen species are exacerbated and the resulting phenotypes include, reduced activity of enzymes with labile [Fe-S] centers (i.e., ThiH, Acn), increased sensitivity to the superoxide (e.g., growth in PQ), and increased mutation frequency. Increasing

the level of YggX reversed each of these phenotypes. One interpretation of these results is that YggX acts prior to the damage and protects labile [Fe-S] clusters from oxidation. In this scenario, blocking the initial attack on the [Fe-S] clusters would abrogate the above phenotypes (Fig. 6). It is formally possible the YggX acts to remove superoxide or to facilitate GSH independent repair of the oxidized clusters. We observed no increased superoxide dismutase activity in *yggX*\* mutant extracts, and elevated levels of YggX increased the resistance of a wild-type strain (i.e., not limited for GSH) to superoxide suggesting that cluster repair is not the affected step.

[0059] This work and the model described above are consistent with the suggestion that the requirement of *gshA* mutants for the thiazole moiety of thiamine was due to the oxygen lability of the ThiH enzyme (Gralnick, J., et al., supra, 2000). The recent identification of ThiH as a member of a SAM radical protein family is consistent with this notion since members of this family share a motif that is indicative of an oxygen labile [Fe-S] cluster (Sofia, H. J., et al., Nucleic Acids Res. 29:1097-106, 2001; Frey, P. A. and Booker, S., Advances in Free Radical Chem. 2:1-43, 1999). Thus, the characterization of YggX presented here supports our hypothesis that the role of GSH in thiamine synthesis is in repair of the oxidized [Fe-S] cluster in ThiH (Gralnick, J., et al., supra, 2000).

[0060] This work raises a number of provocative questions for future studies. The phenotypes characterized here were the result of relatively high levels of YggX. The conserved location of *yggX* adjacent to *mutY* is intriguing. MutY contains an [Fe-S] center and must function under conditions of oxidative stress to perform its role in repairing oxidatively damaged DNA. Considering results herein, we suggest that YggX protects the [Fe-S] cluster of MutY under conditions of oxidative stress. Although *in vitro* studies on the homologous enzyme Endonuclease III suggest the

[Fe-S] cluster in MutY is not accessible to oxidation (Cunningham, R. P., et al., Biochemistry 28:4450-5, 1989), the need for protection *in vivo* or perhaps during protein folding following synthesis, maturation, and/or conformation changes associated with function are not ruled out.

[0061] The model proposed for the function of YggX *in vivo* encourages us to develop an *in vitro* assay for protection of oxygen labile [Fe-S] clusters. Such *in vitro* experiments may distinguish between various mechanisms that could explain the *in vivo* results and also help frame questions to dissect the possible connection between MutY and YggX functions.

[0062] In summary, our work has provided insight on the function of a previously uncharacterized ORF in *S.enterica*. By the serendipitous use of a strain that was sensitive to the lack of GSH we were able to identify a phenotype associated with increased cellular levels of YggX and offer a plausible model for the role of YggX in cellular metabolism.